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Review

BCL-2 family proteins: changing partners in the dance towards death

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The BCL-2 family of proteins controls cell death primarily by direct binding interactions that regulate mitochondrial outer membrane permeabilization (MOMP) leading to the irreversible release of intermembrane space proteins, subsequent caspase activation and apoptosis. The affinities and relative abundance of the BCL-2 family proteins dictate the predominate interactions between anti-apoptotic and pro-apoptotic BCL-2 family proteins that regulate MOMP. We highlight the core mechanisms of BCL-2 family regulation of MOMP with an emphasis on how the interactions between the BCL-2 family proteins govern cell fate. We address the critical importance of both the concentration and affinities of BCL-2 family proteins and show how differences in either can greatly change the outcome. Further, we explain the importance of using full-length BCL-2 family proteins (versus truncated versions or peptides) to parse out the core mechanisms of MOMP regulation by the BCL-2 family. Finally, we discuss how post-translational modifications and differing intracellular localizations alter the mechanisms of apoptosis regulation by BCL-2 family proteins. Successful therapeutic intervention of MOMP regulation in human disease requires an understanding of the factors that mediate the major binding interactions between BCL-2 family proteins in cells.

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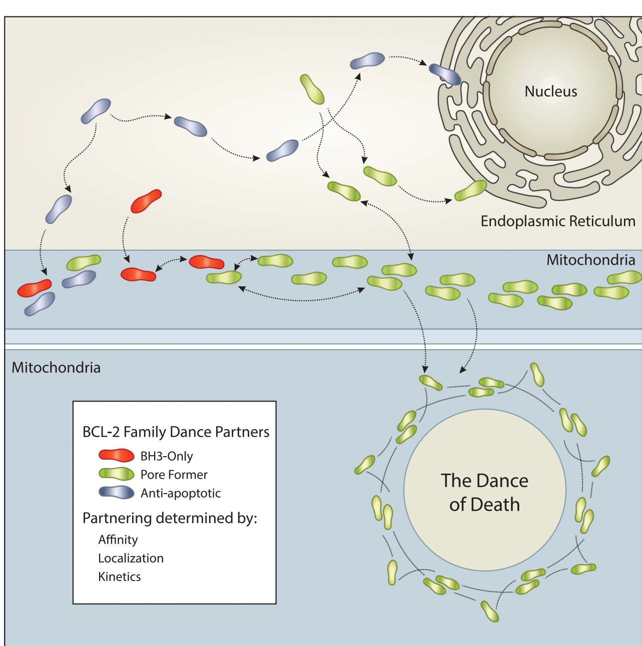

Graphical Abstract

Facts:

- Direct physical interactions between the BCL-2 family proteins dictated by BCL-2 homology 3 (BH3) regions regulate mitochondrial outer membrane permeabilization (MOMP).
- The membrane plays an active role in most BCL-2 family interactions by changing the affinities and local relative abundance of these proteins.
- The majority of studies examining the interactions between BCL-2 family proteins use truncated proteins or peptides of the BH3 region at physiologically irrelevant concentrations or in the absence of membranes leading to confusion in defining the core mechanisms of the BCL-2 family proteins.
- Differential expression in various tissues, targeting to different subcellular localizations and post-translational modifications all contribute to regulation of BCL-2 family-binding interactions.
- Targeting the BH3 domain-binding groove of anti-apoptotic BCL-2 family proteins with BH3 mimetics has proven useful in generating anti-cancer therapeutics but future improvements depend on accounting for more of the factors that govern BCL-2 family interactions.

Questions:

- Other than the BH3 region, which factors determine the dominant interactions between the BCL-2 family proteins?
- How do the affinities between, and physiologically relevant concentrations of, BCL-2 family proteins in cells dictate the regulation of apoptosis?
- How do BAX and BAK transition from inactive monomers to membrane-embedded oligomers that permeabilize membranes?
- How do BCL-2 family proteins control cell death at intracellular locations other than the mitochondria?

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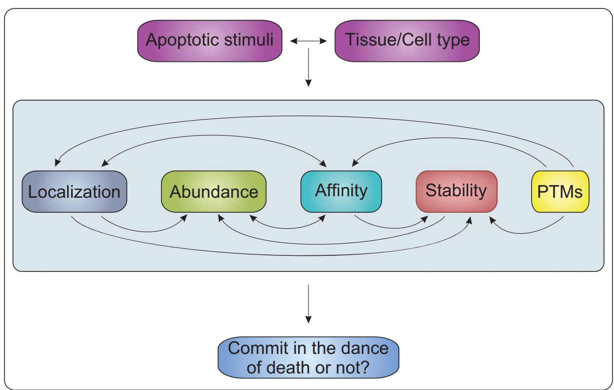


Figure 1 Cellular factors regulating commitment to the apoptotic dance of death by the BCL-2 family proteins. BCL-2 family proteins function by direct binding interactions that lead to mutual sequestration and/or membrane permeabilization. These direct protein–protein binding interactions are functionally regulated in cells by a number of interrelated processes. Differences in apoptotic stimuli and cell types lead to different responses due to the integration of the effects of localization, abundance, affinity, stability and post-translational modifications (PTMs). Related interactions are connected by arrows. For example, PTMs affect protein stability, localization and binding affinities. Affinities determine which interactions dominate but also affect localization and stability (heterodimerization with BH3-only proteins stabilizes MCL-1). The affinities of BCL-2 family proteins for intracellular membranes and other binding partners at these membranes dictate BCL-2 family localization. Co-localization increases local concentrations (abundance). Protein stability also impacts abundance of the BCL-2 family proteins. The relative abundance and affinity ultimately determine which binding interactions dominate and whether or not the cell undergoes MOMP committing it to apoptosis

Introducing the dance of death

Dancing is a joy of life; however, cells have a dance between the BCL-2 family proteins that can lead to death. Exchange of dance partners within the BCL-2 family proteins regulates apoptosis. The outcome of this dance – the fate of the cell – is decided by the net interactions between the BCL-2 family proteins. These interactions are governed by the abundance of the proteins and the ‘attraction’ (affinity) between the partners. The affinities between the dance partners are the result of protein conformation changes, most of which occur on the dance floor – the intracellular membranes where the interactions between the BCL-2 family proteins take place. Further, the apoptotic stimuli, cell type and post-translational modifications differentially regulate the interactions that predominate to control MOMP. This concept of context-dependent regulation of apoptosis is outlined in Figure 1.

In one view, the ultimate goal of the dance between the BCL-2 family proteins is to trigger apoptosis by forming pores within the mitochondrial outer membrane. MOMP results in the release of pro-apoptogenic factors (e.g., cytochrome *c*) from the mitochondrial intermembrane space (IMS) into the cytosol ultimately causing the activation of a caspase cascade that functions to dismantle and destroy the cell.[1] Caspases cleave hundreds of proteins vital for proper cellular function and homeostasis culminating in the typical biochemical and morphological features of apoptosis.[2]

Commitment to the apoptotic dance of death is due in part to MOMP being rapid and complete – cytochrome *c* is released from most mitochondria within 5 mins and complete caspase activation occurs within 15 minutes inevitably resulting in cell death.[3–5] As BCL-2 family proteins regulate this critical cell fate decision an abundance of research has focused on understanding the interactions between the BCL-2 family proteins, the regulation of these interactions within a cell, and how these interactions lead to MOMP.

Dancing on and within membranes – molecular mechanisms of BCL-2 family regulation

The BCL-2 family is divided into three groups based on their primary function (1) anti-apoptotic proteins (BCL-2, BCL-X_L, BCL-W, MCL-1, BFL-1/A1), (2) pro-apoptotic pore-formers (BAX, BAK, BOK) and (3) pro-apoptotic BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, NOXA, PUMA, etc.). All BCL-2 family proteins contain a BH3 domain; one of four BH domains involved in interactions between these proteins.[6] The anti-apoptotic and pore-forming proteins contain all four BH domains (multi-BH domain proteins) and adopt a highly conserved tertiary structure forming a hydrophobic BH3 domain-binding groove that acts as a receptor for BH3 domains of other family members.[7] The BH3-only proteins are subdivided into activator and sensitizer proteins, which contain only the BH3 domain and with the exception of BID are unstructured in solution, reviewed in ref. 8. With the stage set and the dancers named, who ‘dances’ with whom dictates whether or not MOMP occurs.

Various competing models (reviewed in ref. 9) describe how BCL-2 family proteins interact with each other to control MOMP. Figure 2 highlights the ‘embedded together’ model that puts an emphasis on the role of the membrane as the ‘locus of action’ for most BCL-2 family proteins.[10] In all models, the BH3 domain is necessary for the primary apoptotic function of BCL-2 family members and the interactions between them at intracellular membranes. The BH3 domain of activator BH3-only proteins binds to the BH3 domain-binding groove in BAX/BAK.[11,12] This activates BAX and BAK, eliciting a series of conformation changes that result in BAX/BAK homo-oligomerization and pore formation within the MOM (Figure 2b). The BH3 domain-binding groove in anti-apoptotic proteins binds the BH3 domains of the pore-formers and the activator BH3-only proteins, inhibiting their function by sequestering them (Figure 2c).[13,14] The BH3 domain of sensitizer BH3-only proteins binds to the BH3 domain-binding groove of anti-apoptotic proteins, inactivating them.[15] This interaction is competitive with binding of activator BH3-only proteins and pore-formers leading to their displacement from anti-apoptotic proteins (Figures 2d and e). Many, if not most, of these interactions occur at, on and within the MOM.[16] The lipid bilayer has an active role in facilitating structural changes of the BCL-2 family proteins that alter their affinities and consequently the interactions between them thus governing whether or not MOMP occurs.[17]

The embedded together model is more complicated than the original rheostat model that proposed apoptotic fate is determined by the ratio of pro-apoptotic to pro-survival proteins in a cell.[18] The rheostat model was conceived when the BCL-2 family consisted of BCL-2 and BAX and posited that the more abundant protein decided life versus death, respectively. In the context of the more complicated interactions between the expanded families of BCL-2 proteins the

basic posit of the rheostat model remains relevant. Ultimately, the fate of the cell is decided by the interactions between the BCL-2 family dancers. The interactions are determined by the relative abundance and affinities of the partners. Both are impacted by the affinity for membranes, which regulates the localization, conformation and therefore, function of BCL-2 family members. However, similar to the rheostat model, if the sum of interactions results in BAX/BAK oligomers and MOMP– then the cell is committed to death and will dance no more.

Attraction among the BCL-2 family proteins dictates dance partners

Understanding the primary interactions that occur between the BCL-2 family members requires knowledge of the affinity and concentration of each member. These factors dictate the predominate interactions and thus if MOMP occurs. The consequences of differing affinities between the BCL-2 family proteins can be illustrated with a simple case of binding competition between cBID, BCL-X_L and BAX. We have previously demonstrated that BCL-X_L functions as a dominant negative BAX.[19] When cBID, BCL-X_L and BAX were incubated together, BAX-mediated membrane permeabilization was completely inhibited. If a mutant of cBID that interacts with BAX but not BCL-X_L was used, BAX-mediated membrane permeabilization was not inhibited to the same extent. This suggests that cBID preferentially interacts with BCL-X_L over BAX. Using purified full-length recombinant BAX, BCL-X_L and cBID we have found that the affinity (dissociation constant, K_D) between BCL-X_L and cBID is 3 nM (unpublished data), whereas the affinity between cBID and BAX is high micromolar in the absence and 25 nM in the presence of membranes.[20] The interaction between cBID and BAX in the absence and presence of BCL-X_L can be modeled using these K_D values (Figure 3a).[21] The higher affinity of cBID for BCL-X_L versus that of BAX prevents BAX activation and only a supraphysiological concentration of BAX can out-compete BCL-X_L for cBID binding. This simple example does not account for interactions between BAX and BCL-X_L, the differing affinities of the proteins for membranes, changing conformations within the bilayer or the addition of sensitizer BH3-only proteins like BAD. The situation becomes more complex in cells where BCL-2 family proteins interact with known and unknown binding partners at different subcellular locations. Transcriptional and post-translational control of the BCL-2 family proteins further changes the abundance and affinities of these proteins modifying their interactions and localizations within the cell. These factors result in a complex interaction network, the sum of which determines cellular fate.

The first step in understanding the BCL-2 family interaction network is to establish the affinities between binding partners. The reported affinities of known BCL-2 family protein interactions are summarized in (Tables 1A and 1B). Most of the affinity data were generated with BH3 peptides and c-terminally truncated multi-BH domain proteins. Organizing this affinity data by BH3 peptide length in Tables 1A and 1B, revealed a distressing trend. The length of BH3 peptides used in the literature varies from 16 amino acids to >30. As observed for BAK, and BAD, the BH3 peptide length can have a large effect on the measured affinity.[22,23] Adding to the confusion, different peptide lengths are often used for BH3-only proteins within the same paper.[24,25] Generally, BH3 peptide length correlates with increased affinity for the binding partner suggesting that residues outside of the BH3 domain facilitate proper interactions among BCL-2 family proteins.[26] This summary found BIM to be the most studied BH3 peptide but also revealed a scarcity in studies that measure affinities for BAX or BAK binding. In fact, little data have been reported for many of the BH3 proteins including BAP31, BCL-B, BCL-G, BCL-RAMBO, BCL-W, BECLIN-1, BFK, BFL-1/A1, BOK and SPIKE binding to multi-BH domain proteins.

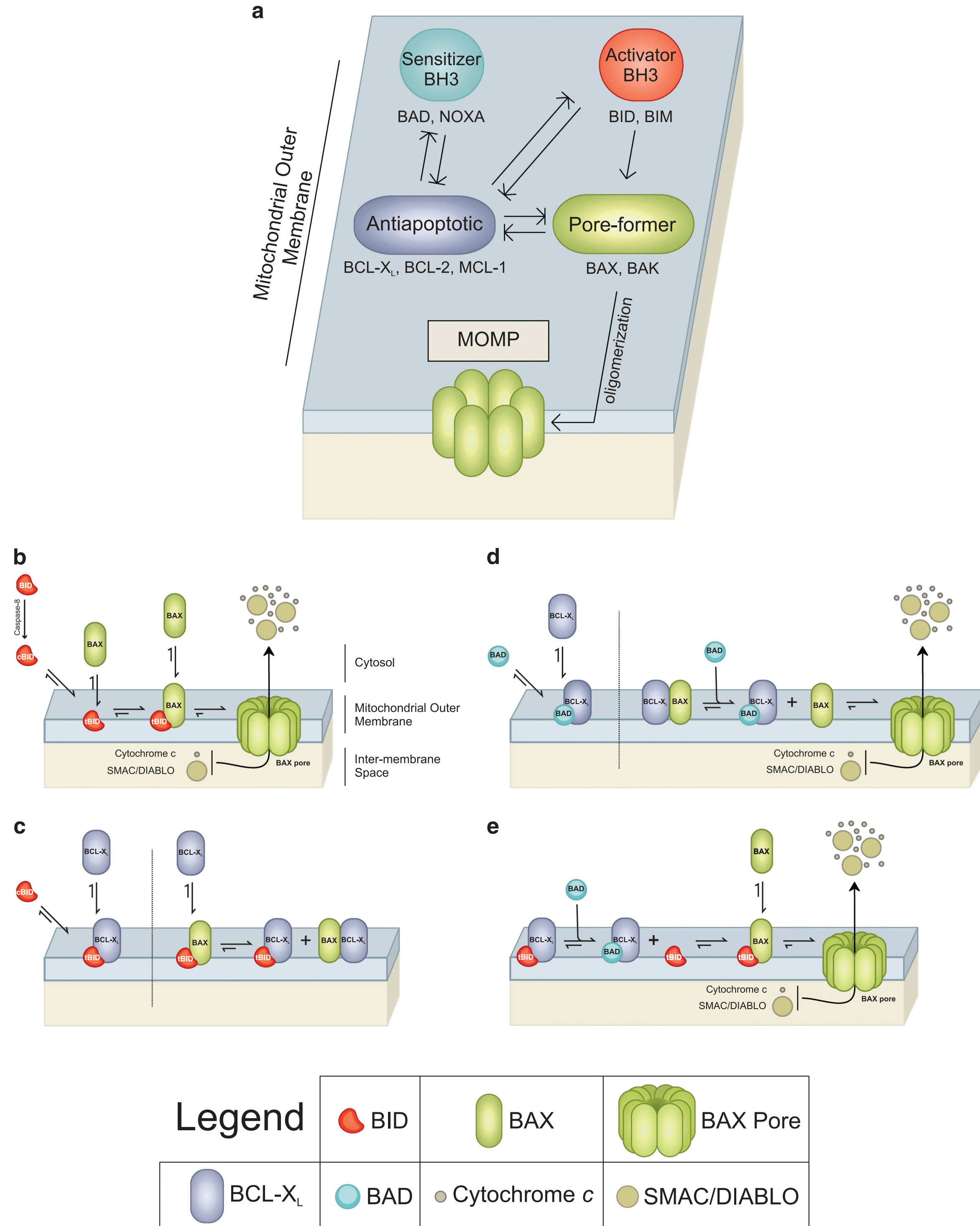
To understand the reported affinities in a cellular context, the physiologically relevant concentration ranges for BCL-2 family proteins must be known. It is frequently stated that cellular concentrations of BCL-2 family proteins are in the low nanomolar range. However, with the exception of BAX, with endogenous levels reported between 3 nM and 170 nM, it appears that the low nanomolar statements are not well supported by data.[27,28] Direct measurement of concentrations in cells is highly desirable as the affinities between full-length BCL-2 family proteins are in the low nanomolar range. Thus, the micromolar dissociation constants measured using peptides could be irrelevant if other BCL-2 family protein concentrations are in the low nanomolar range like BAX.

Studies with full-length proteins can reveal physiologically relevant interactions previously overlooked owing to the use of peptides. For example, many studies state that NOXA binds MCL-1 and BFL-1/A1 but does not bind BCL-2, BCL-X_L or BCL-W, whereas BAD binds BCL-2, BCL-X_L and BCL-W but not MCL-1 or BFL-1/A1.[29,30] This matches well with the reported affinities for NOXA and BAD peptides binding truncated anti-apoptotic proteins (Tables 1A and 1B). NOXA has nanomolar affinity for MCL-1 and BFL-1/A1 and micromolar affinity (which may not be relevant physiologically) with BCL-2, BCL-X_L or BCL-W, whereas BAD is the reverse. However, these affinities and binding specificities change when full-length proteins are used. Full-length NOXA bound with dissociation constants of 3.4 nM for MCL-1, 70 nM for BCL-X_L and 250 nM for BCL-2, demonstrating full-length NOXA binding selectivity but not specificity.[31] Consistent with these affinities, NOXA and BCL-2 interactions contributed to drug resistance in lymphoid cells suggesting that BCL-2 can prevent NOXA-mediated apoptosis.[31]

Most methods used to measure protein–protein interactions between BCL-2 family proteins neglect the influence of membranes. For example, full-length cBID and BAX interact in the presence of lipid membranes but not in solution because cBid requires a conformation change at membranes to interact with and activate BAX.[17,20] A complete map of these interactions with affinities of full-length proteins in solution and in membranes will be essential to the delineation of the interactions between BCL-2 family proteins that regulate cell death.

Defining roles in the dance – what factors make a BH3-only protein an activator?

BH3-only proteins are classified as activator or sensitizer proteins based on whether or not they bind to and activate BAX and BAK. Activator BH3-only proteins promote MOMP



directly by triggering oligomerization of BAX and BAK. In contrast, sensitizer BH3-only proteins promote MOMP indirectly via binding to and inhibiting the anti-apoptotic proteins. However, activator BH3-only proteins bind both BAX and BAK and anti-apoptotic proteins. Furthermore, binding of an activator BH3-only protein to an anti-apoptotic protein also inhibits the anti-apoptotic protein. Therefore, who is inhibiting whom, comes down to the relative abundance and affinities between activator BH3-only proteins and anti-apoptotic proteins. This redefines the interaction between activators and anti-apoptotic proteins as 'mutual sequestration', whereby BH3-only proteins bind to and inhibit anti-apoptotic proteins and vice versa.[9] Furthermore, activator BH3-only proteins promote the membrane-embedded

Figure 2 The dance of the BCL-2 family within the bilayer regulates mitochondrial outer membrane permeabilization (MOMP) and apoptosis (**a**) Schematic of the embedded together model. All binding interactions are reversible and equilibria are governed by local affinities. Interactions with the lipid bilayer change the affinities of the interactions and therefore have an active role in the functions of the proteins. Binding of the activator BH3-only proteins (e.g., BID, BIM) to membranes increases their affinity for the pore-formers (e.g. BAX, BAK), which are activated (arrows) to permeabilize the mitochondrial outer membrane. The anti-apoptotic proteins (e.g., BCL-X_L, BCL-2, MCL-1) inhibit both the activator BH3-only proteins and the pore-forming proteins by mutual sequestration (T'd arrows). The sensitizer BH3-only proteins (e.g., BAD, NOXA) bind to and inhibit the anti-apoptotic proteins also by mutual sequestration. Recruitment of the complexes to the membrane by constitutive interactions (e.g., BAK) and dynamic interactions (e.g., BAX, BID, BIM) increases the affinities and local concentrations and reduces the diffusion of the BCL-2 family proteins. Localization at different intracellular membranes also dictates the binding equilibria between each family member. The efficiency of inhibition by mutual sequestration of anti-apoptotic proteins depends on both affinities and off-rates of the interactions. Interaction of the BH4 region of the anti-apoptotic proteins with BAX shifts the BAX-membrane binding equilibrium to favor the unbound state (retrotranslocation, not shown). (**b–d**). Interactions of the BCL-2 family that promote or inhibit MOMP illustrated for cBID. BAX, BCL-X_L and BAD as examples of different functional categories. (**b**) BID is activated by caspase-8 mediated cleavage to cBID (cleaved BID) a protein comprised of two fragments BID-P7 and BID-P15 held together by hydrophobic interactions. Rapid high-affinity binding to membranes dissociates the p7 fragment to solution and favors insertion of the p15 fragment (tBID; truncated BID) into the membrane. Membrane-bound tBID recruits inactive BAX from the cytosol. Binding to tBID activates BAX to insert in the bilayer, oligomerize and permeabilize the mitochondrial outer membrane releasing intermembrane space proteins including cytochrome *c* and SMAC. (**c**) Active tBID and BAX can recruit BCL-X_L to the membrane resulting in inhibition of both pro and anti-apoptotic proteins by mutual sequestration. BCL-X_L prevents tBID from activating BAX and prevents BAX from oligomerizing resulting in the inhibition of MOMP. BAX bound to BCL-X_L is in the active (oligomerization competent) conformation. (**d**, **e**) BAD inhibits unbound BCL-X_L by mutual sequestration. The affinity of BCL-X_L is higher for tBID than for active BAX (Tables 1A and 1B) therefore, in the absence of other regulatory interactions or PTMs if BCL-X_L is bound to tBID and BAX then high concentrations of BAD will displace active BAX (**d**) and then tBID (**e**) from BCL-X_L resulting in MOMP

conformation of anti-apoptotic proteins and thus also 'activate' these proteins.[19] As a result a BH3-only protein with lower affinity for the membrane-bound form of an anti-apoptotic protein may function primarily to activate it. At high relative concentrations activator BH3-only proteins are expected to inhibit anti-apoptotic proteins while also activating BAX/BAK. For example, mutants of both BIM and BID that cannot bind BAX but can bind anti-apoptotic proteins promote apoptosis by functioning as sensitizer BH3-only proteins.[32–34] Similarly, specific mutations to the BH3 domains of sensitizer BH3-only proteins BAD and NOXA turn these proteins into direct BAX activators without abrogating interactions with BCL-X_L.[12] Therefore, an activator BH3-only protein is a sensitizer-protein that has gained an additional dance partner aiding in the dance of death through activation of BAX or BAK.

Does the capacity to bind and activate pore-formers constitute the 'activator' classification of a BH3-only protein? Many BH3 peptides bind both anti-apoptotic proteins and pore-formers owing to the similarity between the BH3 domain-binding grooves. For example, the BH3 sensitizer NOXA primarily targets MCL-1 with an affinity of 3.4 nM.[31] However, both NOXA and the *bona fide* activator BH3, BID can activate BAX to permeabilize membranes *in vitro,* causing NOXA to be classified as an activator BH3-only protein in some reports.[35,36] This confusion is cleared up by examining affinities; the affinity of BID to BAX is 25 nM, whereas the affinity of NOXA to BAX is 1000 times higher (25 μM) (Figure 3b). BAX can be activated by NOXA, however, physiologically irrelevant micromolar concentrations are required. Therefore, the capacity to bind and activate pore-formers at physiologically relevant concentrations defines a BH3-only protein as an activator.

The question now is: what confers activator function and an increased affinity to BAX/BAK for full-length BH3-only proteins compared with peptides? The BH3 domain has a significant role in determining binding affinity, and mutations to the BH3 domains of BID and BIM fully abrogate their activator function.[12] However, the BH3 domain is not the only portion of the protein promoting BAX/BAK activation. Low nanomolar ($<$10 nM) concentrations of recombinant full-length BID or BIM activate BAX *in vitro*, whereas micromolar concentrations of BH3 peptides are needed to achieve the same level of BAX activation.[37] The creation of SAHBs (Stabilized Alpha-Helices of BCL-2 domains) highlighted the importance of BH3 domain secondary structure.[38] SAHB peptides are forced into a tight alpha helical structure through a 'staple' – a covalent bond between two regions of the peptide. SAHBs of BID and BIM are more efficacious in BAX activation compared with their 'unstapled' BH3 peptides but still require 200–600 nM concentrations to fully activate BAX.[39,40] Thus, an alpha helical BH3 domain is not enough for full efficacy.

High nanomolar concentrations of helical BH3 domain peptides may be required because the peptides do not bind to membranes as do cBID and BIM.[41–43] Tethering a BID BH3 peptide, or a BID SAHB to the membrane resulted in efficient BAX activation at low nanomolar concentrations albeit with slower membrane permeabilization kinetics compared with full-length tBID.[40,44] Membrane targeting of the BID BH3 peptide increases the alpha-helicity of the BH3 domain such that the peptide activates BAX as efficiently as a membrane-targeted SAHB.[40,44] This indicates two things (1) binding to membranes increases the alpha-helicity of the BH3 domain increasing affinity for BAX and (2) membrane binding likely increases the activity of these peptides by increasing the local concentration of the peptide resulting in more efficient BAX activation. However, membrane-targeted peptides are still 5–10 times less efficacious at activating BAX compared with full-length proteins, suggesting other regions outside the BH3 domain increase the affinity between activator BH3-only proteins and BAX/BAK.[40]

The dance steps of the pore-formers – mechanisms of BAX/BAK-mediated MOMP

The BAX and BAK activation mechanism is a multi-step dance within the bilayer. We and others have delineated steps in the BAX/BAK activation pathway that are generally agreed upon.[20,45–47] Activator BH3-only proteins contribute to recruitment of BAX to membranes, activating BAX/BAK monomers, which then undergo a substantial conformation change and embed within the bilayer. Activated BAX/BAK monomers form symmetric homodimers that oligomerize to form pores within the bilayer resulting in MOMP. Understanding these steps is imperative because each represents an opportunity for therapeutic intervention in human disease. Several studies have focused on determining the exact conformations of inactive and

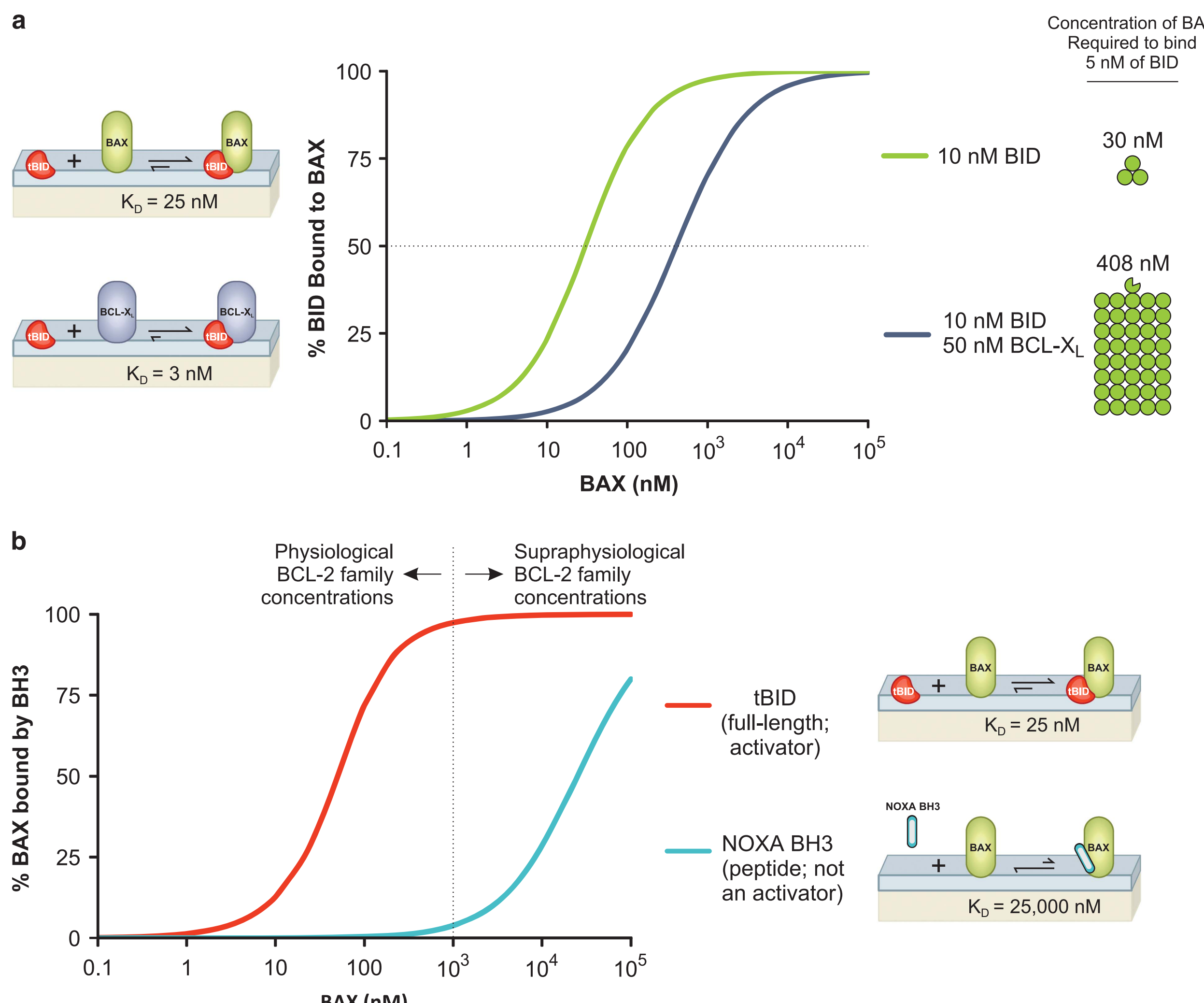


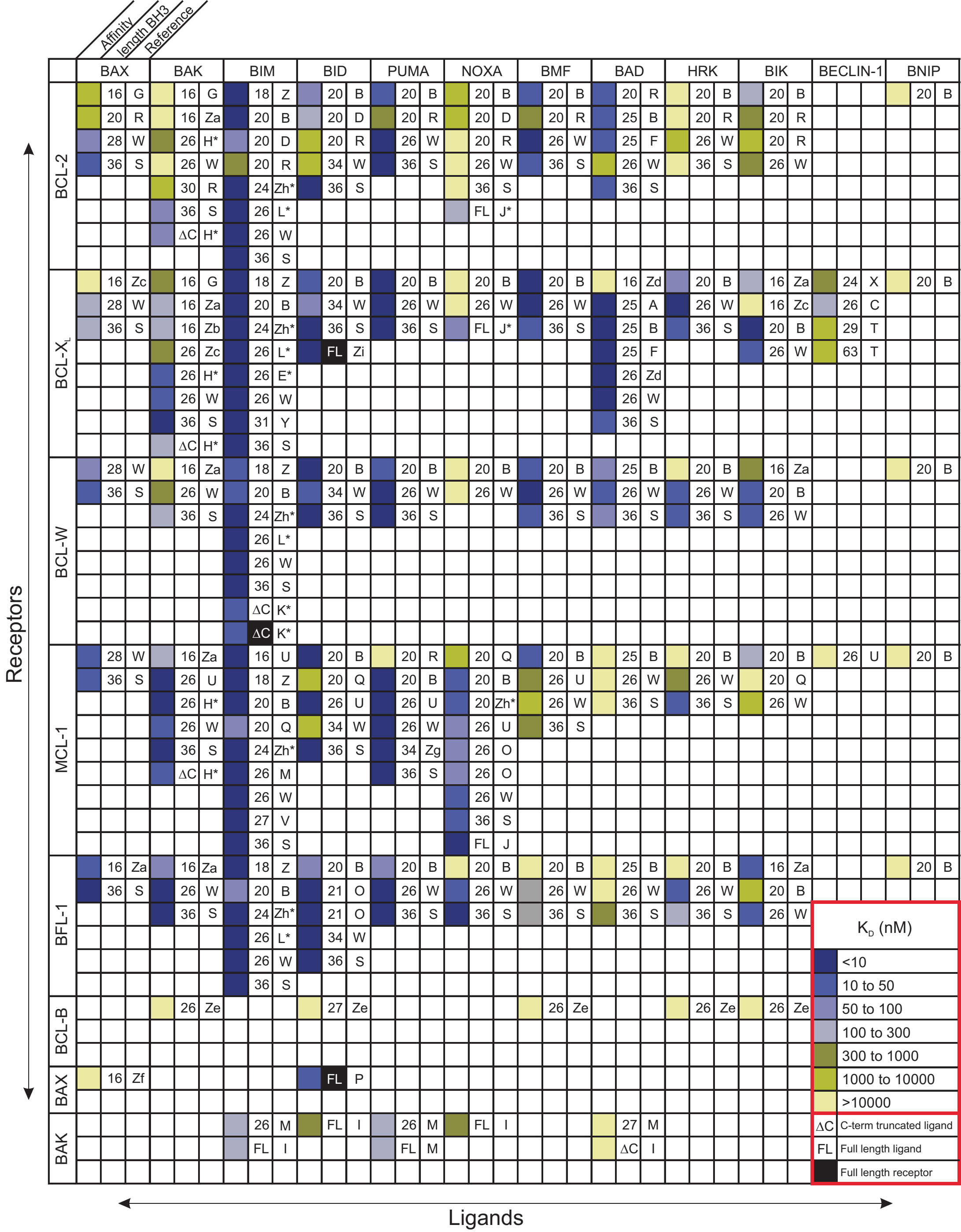
Figure 3 Affinities and concentrations dictate the predominate interactions of the BCL-2 family members (**a**) At membranes cBID binds BAX and BCL-X_L with an affinity of 25 nM and 3 nM, respectively. Equations describing competitive binding of two different 'ligands' (BAX, BCL-X_L) to one protein molecule (cBID)[21] were used to model the Interaction of 10 nM cBID with increasing concentrations of BAX in the absence (green) and presence (purple) of 50 nM BCL-X_L. In the absence of BCL-X_L, 50% of 10 nM cBID is bound to BAX at a BAX concentration of 30 nM. Upon the addition of BCL-X_L, ~ 13 times more BAX (408 nM) is required to bind 50% of the cBID. In this case, BCL-X_L is binding the majority of the cBID, preventing BAX activation. The functional consequence of the differing affinities results in BCL-X_L out-competing BAX for binding to cBID, effectively inhibiting apoptosis at physiologically relevant concentrations of BAX. (**b**) The affinities between BH3-only proteins and BAX determine whether a BH3-only protein functions as an activator or sensitizer. Equations describing the binding of two proteins[21] were used to model the interaction of 50 nM BAX with increasing concentrations of the indicated BH3-only protein/peptide; full-length tBID (red) and NOXA BH3 peptide (cyan).The affinities of full-length tBID or NOXA BH3 peptide for BAX are 25 nM[20] and 25 000 nM (estimated from ref. 35) respectively. Typical *in vitro* BAX activation assays use peptide concentrations in the micromolar range, well above the nanomolar concentrations predicted for BCL-2 family proteins in cells. At supraphysiological concentrations (>1000 nM) of peptides (e.g., NOXA, cyan) some BH3 sequences that typically function as sensitizers can bind to and activate BAX

active BAX/BAK, how their affinities for BCL-2 family proteins change and the transitions between conformations that occur for BAX/BAK to elicit MOMP. Despite years of examination, the complex mechanism is not fully understood partly owing to the difficulties in studying membrane-embedded proteins.

The specifics of BAX/BAK conformations during their activation and how they form pores have been reviewed recently.[45,47] Rather than re-examine the precise conformations of BAX/BAK we will highlight some assumptions and unknowns regarding these models and how steps are regulated by changes in binding partners, affinities and conformations within the bilayer (Figure 4).

Activator BH3-only proteins start the dance of death by interacting with BAX/BAK at the bilayer. Activator BH3-only proteins interact with BAX/BAK at the bilayer owing to the high affinity of activator BH3-only proteins for membranes (Figure 4; step 1). For BAK, it is obvious that this interaction occurs at the bilayer because BAK is constitutively localized to the MOM. However, many models assume that BAX interacts with activator BH3-only proteins in solution resulting in a soluble activated BAX that then inserts into the MOM.[47–49] The data supporting interactions between activator BH3-only proteins and BAX in solution are from experiments using truncated BAX, BH3 peptides or

Table 1A BCL-2 family interactions; dissociation constants reported for BH3 peptides, truncated and full-length proteins



Receptors	BAX	BAK	BIM	BID	PUMA	NOXA	BMF	BAD	HRK	BIK	BECLIN-1	BNIP
BCL-2	16 G	16 G	18 Z	20 B	20 B	20 B	20 B	20 R	20 B	20 B		20 B
	20 R	16 Za	20 B	20 D	20 R	20 D	20 R	25 B	20 R	20 R		
	28 W	26 H*	20 D	20 R	26 W	20 R	26 W	25 F	26 W	20 R		
	36 S	26 W	20 R	34 W	36 S	26 W	36 S	26 W	36 S	26 W		
		30 R	24 Zh*	36 S		36 S		36 S				
		36 S	26 L*			FL J*						
		ΔC H*	26 W									
			36 S									
BCL-XL	16 Zc	16 G	18 Z	20 B	20 B	20 B	20 B	16 Zd	20 B	16 Za	24 X	20 B
	28 W	16 Za	20 B	34 W	26 W	26 W	26 W	25 A	26 W	16 Zc	26 C	
	36 S	16 Zb	24 Zh*	36 S	36 S	FL J*	36 S	25 B	36 S	20 B	29 T	
		26 Zc	26 L*	FL Zi				25 F		26 W	63 T	
		26 H*	26 E*					26 Zd				
		26 W	26 W					26 W				
		36 S	31 Y					36 S				
		ΔC H*	36 S									
BCL-W	28 W	16 Za	18 Z	20 B	20 B	20 B	20 B	25 B	20 B	16 Za		20 B
	36 S	26 W	20 B	34 W	26 W	26 W	26 W	26 W	26 W	20 B		
		36 S	24 Zh*	36 S	36 S		36 S	36 S	36 S	26 W		
			26 L*									
			26 W									
			36 S									
			ΔC K*									
			ΔC K*									
MCL-1	28 W	16 Za	16 U	20 B	20 R	20 Q	20 B	25 B	20 B	20 B	26 U	20 B
	36 S	26 U	18 Z	20 Q	20 B	20 B	26 U	26 W	26 W	20 Q		
		26 H*	20 B	26 U	26 U	20 Zh*	26 W	36 S	36 S	26 W		
		26 W	20 Q	34 W	26 W	26 U	36 S					
		36 S	24 Zh*	36 S	34 Zg	26 O						
		ΔC H*	26 M		36 S	26 O						
			26 W			26 W						
			27 V			36 S						
			36 S			FL J						
BFL-1	16 Za	16 Za	18 Z	20 B	20 B	20 B	20 B	25 B	20 B	16 Za		20 B
	36 S	26 W	20 B	21 O	26 W	26 W	26 W	26 W	26 W	20 B		
		36 S	24 Zh*	21 O	36 S	36 S	36 S	36 S	36 S	26 W		
			26 L*	34 W								
			26 W	36 S								
			36 S									
BCL-B		26 Ze		27 Ze			26 Ze		26 Ze	26 Ze		
BAX	16 Zf			FL P								
BAK			26 M	FL I	26 M	FL I		27 M				
			FL I		FL M			ΔC I				

The affinity of a protein interaction is generally reported as a dissociation constant (K_D). Unless otherwise specified, K_D values obtained from the literature are for interactions between truncated multi-BH region protein 'receptors' (rows) and the corresponding BH3 'ligand' (columns). For each BH3 ligand, the first column indicates the K_D represented on a color scale (legend bottom right of chart). The second column indicates the length of the BH3 peptide used. 'ΔC' represents a BH3-only protein lacking its C-terminal tail anchor and 'FL' indicates full-length BH3-only protein. Use of a full-length multi-BH region protein 'receptor' is indicated by black shading in the second column. The letter in the third column indicates the PMID and method, listed in B. For each interaction, K_D values were sorted by peptide length (increasing length downward across rows). A reference with an asterisk (*) indicates the original report indicated that detergent was present when the K_D was measured

Table 1B PubMed ID number and method used to measure the K_D values listed in A

Label	PMID	Method
A	11206074	Fluorescence polarization (FP)
B	16697956	
C	17446862	
D	12242151	
E	19748896	
F	11248023	
G	11904405	
H	19351886	Surface plasmon resonance (SPR)
I	21727192	
J	21454712	
K	12660157	
L	15694340	
M	24265320	
N	22156224	SPR and time-resolved (TR)-FRET
O	21395401	
P	19062087	Forster resonance energy transfer (FRET)
Q	20392693	Isothermal titration calorimetry (ITC)
R	23996493	
S	21060336	
T	17337444	
U	18589438	
V	17389404	
W	28411240	
X	18641390	
Y	20363230	Yeast surface display
Z	23363053	
Za	21713285	Bacterial surface display
Zb	19766123	
Zc	9020082	Tryptophan fluorescence quenching
Zd	9372935	
Ze	23192964	NMR spectroscopy
Zf	24434006	
Zg	23301700	Stopped flow technique
Zh	25052212	Fluorescence anisotropy
Zi	27108441	FRET

detergents that promote the conformational changes of BAX that normally occur at a lipid bilayer. Indeed, activator BH3 peptides remain in solution when binding to BAX/BAK, however, micromolar amounts of activator are required to fully activate BAX/BAK with slow membrane permeabilization kinetics compared with activation with full-length activator BH3-only proteins.[37,40] In comparison, full-length activator BH3-only proteins have a high affinity for membranes and efficiently activate BAX/BAK at nanomolar concentrations resulting in rapid membrane permeabilization kinetics.[20,35,37,40] Activator BH3-only proteins bind membranes (half-time <50 s) before interacting with BAX (half-time$\approx$500 s).[50] Furthermore, activator BH3-only proteins require membrane binding for efficient BAX/BAK-mediated membrane permeabilization *in vitro* and in cells.[20,41,51] Thus, binding between activator BH3-only proteins and BAX/BAK in solution may reflect what can happen with these proteins but in cells the relevant interactions occur at and within intracellular membranes.

Inviting BAX and BAK to dance without activator BH3-only proteins. BAX or BAK can be activated by antibodies,[52] small molecules,[53,54] heat,[55] pH,[56] detergents[57–59] and metabolites,[60] suggesting that all of the information for oligomerization and pore formation is contained within BAX and BAK. Thus, spontaneous activation of BAX/BAK could occur due to any perturbation of their compact globular structure that results in exposure of their hydrophobic cores to membranes. In line with these data, HCT116 cells with a genetic loss of 8 known BH3-only proteins (BAD$^{-/-}$, BID$^{-/-}$, BIK$^{-/-}$, BIM$^{-/-}$, BMF$^{-/-}$, HRK$^{-/-}$, NOXA$^{-/-}$ and PUMA$^{-/-}$) underwent apoptosis in a BAX/BAK-dependent manner once anti-apoptotic BCL-2 proteins were genetically or pharmacologically inactivated.[61] Further, exogenous expression of either BAX or BAK in cells lacking the majority of BCL-2 family proteins (the above HCT116 cells lacking 8 BH3-only proteins with additional genetic loss of BCL-2, BCL-XL, MCL-, BCL-W, BFL/A1, BNIP3, NIX, BAX and BAK) resulted in spontaneous targeting of BAX and BAK to mitochondria and subsequent apoptosis, suggesting that BAX and BAK do not necessarily require BH3-only proteins for activation *in vitro*.[61] However, it must be kept in mind that cultured cells are abnormal, under stress owing to high oxygen levels, often transformed and are usually growing on plastic potentially altering the cytoplasmic environment, cell metabolism or lipid composition of the mitochondria resulting in conditions that spontaneously activate BAX and BAK.

Dance moves of the pore-formers – activated BAX/BAK undergo multiple conformation changes within the bilayer. BAX and BAK are comprised of nine alpha-helices and in their inactive forms adopt a globular structure.[11,62] Inactive monomers of BAX and BAK are slightly different; the carboxyl-terminal transmembrane helix, α9, is constitutively inserted into the MOM for BAK, whereas for BAX α9 is sequestered to the protein core within the hydrophobic BH3 domain-binding groove resulting in a cytoplasmic localization.[63,64] Activator BH3-only proteins bind to the BH3 domain-binding groove, displacing α9, which then inserts into the bilayer resulting in BAX adopting a similar conformation to that of inactive BAK.[64–66] BAX/BAK disengages the amino (α1) and carboxyl-terminal (α6–α8) regions from the hydrophobic core of the protein that is formed by the BH3 domain-binding groove (α2–α5).[12,67–69] Then BAX/BAK embed into the bilayer with α5 and α6 helices partially inserted and α9, adopting a transmembrane configuration (Figure 4; step 2).[49,67,70–72] Determining whether there is a precise order of these changes and the equilibria between each step is important since a physiologically relevant intermediate conformation of BAX or BAK could be targeted pharmaceutically to modulate the regulation of MOMP. But so far, a defined temporal intermediate between inactive monomeric and membrane inserted dimeric BAX/BAK has not been found in physiologically relevant experimental conditions.

Dancing with a twin – activated monomers of BAX/BAK form symmetric homodimers. Of the BCL-2 family proteins BAX/BAK are unique in that they first form heterodimers with activator BH3-only proteins and then transition by an unclear mechanism to homodimers with other active BAX/BAK monomers (Figure 4; step 3).[73,74] The conundrum arises

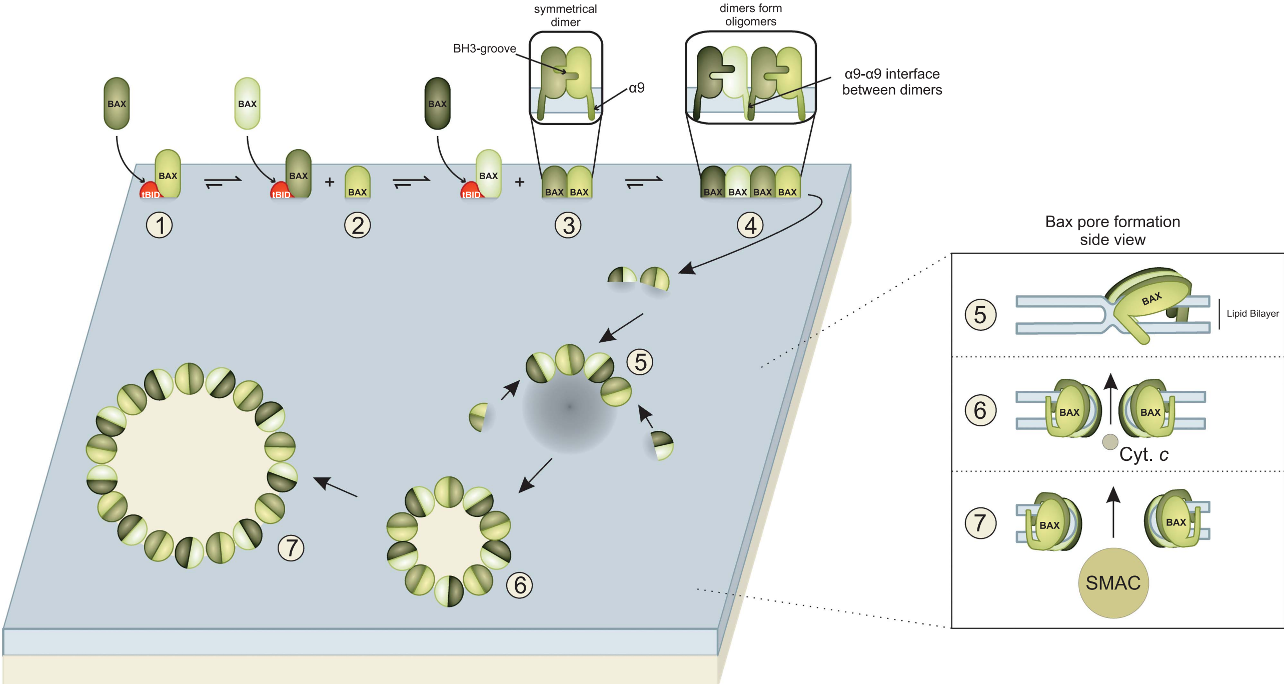


Figure 4 BAX activation is a multi-step process characterized by hetero-and homotypic interactions that result in MOMP. Full-length activator BH3-only proteins, like tBID, have a high affinity for membranes and bind them rapidly before interacting with the pore-formers, BAX and BAK. The following steps are shown for BAX activation; however, the BAK activation mechanism appears to be very similar. (**Step 1**) BAX interacts with tBID at the membrane. (**Step 2**) BAX then undergoes multiple conformation changes, inserting into the bilayer with $\alpha 9$ spanning the membrane. Insertion into the bilayer is the rate-limiting step in the BAX activation mechanism. (**Step 3**) The transition from the BAX and BH3-only protein heterodimer to BAX homodimers is not well understood but would occur spontaneously if the homotypic interaction is of higher affinity than that of the heterotypic interaction between BAX and activator BH3-only proteins. The end result is that the membrane-embedded active BAX monomers dimerize via reciprocal interactions between their BH3-grooves. (**Step 4**) BAX dimers interact with each other via multiple lower affinity interactions of which parallel $\alpha 9$–$\alpha 9$ interactions between dimers appear particularly important for the stabilization of large pores. (**Step 5**) BAX oligomers composed of symmetrical dimer subunits start to destabilize and thin the bilayer. Weak affinity interactions between dimers allow additional dimer subunits to add to the oligomer at any point. (**Step 6**) BAX oligomers form small pores in the bilayer that can initially release smaller intermembrane space (IMS) proteins like cytochrome *c* (12 kDa)**.** (**Step 7**) Continuing activation of BAX results in a higher concentration of dimers in the bilayer that add to the oligomer, resulting in pore expansion and the release of larger IMS proteins like SMAC (54 kDa dimer)

because the BH3 domain of the activator BH3-only protein binds inactive BAX/BAK in the BH3 domain-binding groove and BAX/BAK dimerization involves symmetric binding between these BH3 domain-binding grooves, which also contain the BH3 domain, of each activated monomer.[12,67,71,72,75] With similar interaction sites, activator BH3-only proteins would be expected to inhibit rather than activate BAX/BAK dimerization, which has not been observed for BAX/BAK even in the presence of excess activator.[36,76] In support of this, BID SAHBs, previously shown to activate BAK, inhibited BAK oligomerization when disulfide cross-linked to the BAK BH3 domain-binding groove.[11] This suggests that activator BH3-only proteins leave the BH3 domain-binding groove in order for BAX/BAK oligomerization to proceed. However, at steady state BH3 activators can stay bound to BAX even after pore formation.[20,50,77] These studies measured the entire population of BAX and BID via FRET (Förster resonance energy transfer) thus, it remains possible that there are sub-populations, one of cBID and BAX heterodimers and one of BAX oligomers. It is also possible that binding partners exchange rapidly at equilibrium despite their high affinities.

An attractive, hypothesis for what could be occurring is that at the bilayer, activator BH3-only proteins convert BAX/BAK from inactive monomers into active membrane-embedded monomers.[73] This structural rearrangement could reduce the affinity between activator BH3-only proteins and active BAX/BAK monomers such that BAX/BAK homodimerization displaces the activator BH3-only protein (Figure 4; step 2). Displacement is favored by inactive monomers being higher in concentration and having a higher affinity for the activator BH3-only proteins compared with the active monomers. This would explain why BH3-only proteins function as catalysts for BAX activation in certain experimental conditions.[78] The displaced monomer is limited to two-dimensional diffusion within the bilayer, resulting in a high local concentration of active monomers. This results in the recruitment of multiple activated BAX/BAK monomers in close proximity and thereby assists dimer formation via high-affinity BH3 domain-binding groove interactions (Figure 4; step 3). The high-affinity BH3 domain-binding groove interactions in a dimer along with insertion into the bilayer drive BAX/BAK activation irreversibly towards dimers. Release of the BH3 protein would repeat this process resulting in a high local concentration of BAX/BAK dimers in the bilayer that further oligomerize thereby permeabilizing the membrane (Figure 4; step 4).

Dancing in a circle – low-affinity interactions allow for a dynamic BAX/BAK pore. Fluorescence-based kinetic studies revealed BAX insertion into the bilayers was rate-limiting

and followed rapidly by BAX oligomerization.[20] The last step to be characterized is determining exactly how BAX/BAK dimers dance as oligomers that permeabilize the MOM. Recently, single-molecule imaging of BAX showed BAX monomers form dimers in the bilayer that self-assemble into higher-order oligomers.[73] This is consistent with biochemical data where BAX/BAK symmetrical homodimers can be disulfide cross-linked into higher-order oligomers[71,74] and with structural data of active BAX/BAK at membranes.[67,72] In addition, the BAX/BAK inhibitors MSN-125, MSN-50 and DAN004 prevent BAX oligomerization but not symmetrical dimer formation.[79] These inhibitors prevented some but not all of the interfaces within symmetrical dimers suggesting that proper dimer formation is prerequisite for oligomerization and pore formation.

Chemical cross-linking studies report inter-dimer cross-links at a number of positions between one BAX molecule in a dimer ($\alpha\#^1$) and another in a separate dimer ($\alpha\#^2$) such as $\alpha 6^1{:}\alpha 6^2$, $\alpha 1^1{:}\alpha 1^2$, $\alpha 3^1{:}\alpha 5^2$ and $\alpha 9^1{:}\alpha 9^2$.[71,72,80] The interface formed between α9 helices within the bilayer is particularly important for stabilizing large pores. Deletion of α9 or mutations to α9 that disrupt the inter-dimer interface results in the release of smaller IMS proteins like cytochrome *c* (12 kDa) but not larger proteins like SMAC (54 kDa dimer in cells).[71,81] Consistent with observations that BAX/BAK form dynamic pores that enlarge over time with variable sizes dependent on BAX/BAK concentration,[82,83] direct visualization of BAX in cells revealed that homo-oligomers form lines, arcs and rings of various sizes on mitochondria.[84] In this study 97% of BAX rings formed pores in isolated lipid bilayers versus 12% of BAX arcs. This supports observations that BAX lines the edges of both small and large pores with similar protein density.[85] Together, these data suggest a model whereby oligomers are formed by dimers linked together by many weak affinity interactions (Figure 4; step 4). This model is attractive because weak affinity interactions between dimers explain how pores enlarge over time: newly formed dimers join together end-to-end as lines and arcs that eventually close to form pores that are expanded by additional dimer subunits (Figure 4; step 5–7).

Changing Dance Partners Within the Cell

PTMs alter the abundance and affinities of BCL-2 family proteins in cells. Just as different songs affect dancing styles, post-translational modifications (PTMs) of BCL-2 family proteins have a role in regulating their interactions. PTMs can affect stability, localization and function of BCL-2 family proteins and can promote or inhibit apoptosis. PTMs regulating BCL-2 family proteins were reviewed comprehensively.[86] However, the majority of PTMs are single reports that have not been independently confirmed. In other cases, two or more reports conclude opposite functional effects. For example, phosphorylation of BCL-2 at S70 was reported to increase[87–89] or inhibit apoptosis.[90–92]

To illustrate the range of activities ascribed to post-translational modifications we generated an updated list of PTMs reported for just BAX (Table 2). Even for the well-studied protein BAX there are only two independently confirmed PTMs, polyubiquitination and phosphorylation of S184 of BAX. Polyubiquitination targets BAX for proteasomal degradation. Proteasomal degradation of monomeric BAX would

Table 2 Post-translational modifications of BAX

Modification and residue	Modified by	Functional consequence	Year	PMID
Phosphorylation S184 (H9)	AKT	Inactivates BAX, prevents translocation from cytosol to mitochondria	2004	14766748
			2005	15642728
			2009	19376889
		Activates BAX, promotes mitochondrial localization and MOMP	2016	26763134
			2016	28357332
	PKC-zeta	Inactivates and destabilizes BAX	2007	17525161
Phosphorylation S163 (H8-H9 loop)	GSK-3P	Activates BAX by increased targeting to mitochondria	2004	15525785
Phosphorylation T167 (H8-H9 loop)	JNK and p38	Phosphorylation inactivates BAX Inhibits apoptosis	2006	16709574
Phosphorylation T135 or T140 (H6)	Unknown	Unknown. Observed in a pre-malignant (AT1), but not a malignant cell line (CA1a)	2009	19194518
Phosphorylation T22(H1)	Unknown	HTS, needs validation	2011	21712546
Phosphorylation T85 (H3-H4 loop)	Unknown		2015	25814448
Dephosphorylation T172, T174, T186	WIP1	Downregulates BAX. Inhibits apoptosis	2013	23907458
Dephosphorylation	PP2A	Promotes apoptosis	2006	16679323
Cleavage G28/G29 (H1)	Calpain	Releases C-terminal BAX lacking amino acids 129 of the N-terminus, increases its apoptotic activity by unknown mechanism Promotes apoptosis	2003	12490315
PolyUbiquitination non-specific site (9 lysines in BAX)	unknown	Inhibits apoptosis targets BAX for proteasomal degradation	1998	9438391
			2000	10725400
	PARKIN		2012	22460798
	IBRDC2		2010	20300062

Reported post-translational modifications (PTMs) of BAX. Helix in BAX where modification takes place is indicated (H#). Only phosphorylation of S184 and ubiquitination of BAX have been independently confirmed by multiple labs (shaded). Where the same functional consequence is reported in multiple studies rows were merged for clarity. The functional consequence of S184 phosphorylation is controversial as it has been reported that it either activates or inhibits BAX. The corresponding publication year and Pubmed ID (PMID) are listed for each study

decrease BAX concentration and shift activator BH3 binding to anti-apoptotic proteins (Figure 3a).

Phosphorylation could alter the core mechanism of 'dance' steps detailed above by introducing a large negatively charged phosphate to regions critical for BAX function. Phosphorylation of residue S184 by AKT is known to inhibit BAX.[93,94] Currently, the precise mechanism of how S184 phosphorylation affects BAX activation remains to be elucidated. However, to understand BCL-2 family protein regulation *in vivo* it will be imperative to understand how PTMs change BCL-2 family-binding affinities for proteins and membranes, and their subcellular localizations both of which can alter the fate of the cell.

Alternate dance venues in cells – changing localizations of the BCL-2 family. The BCL-2 family proteins have diverse localizations within cells, likely dictated by their different affinities for various intracellular membranes and

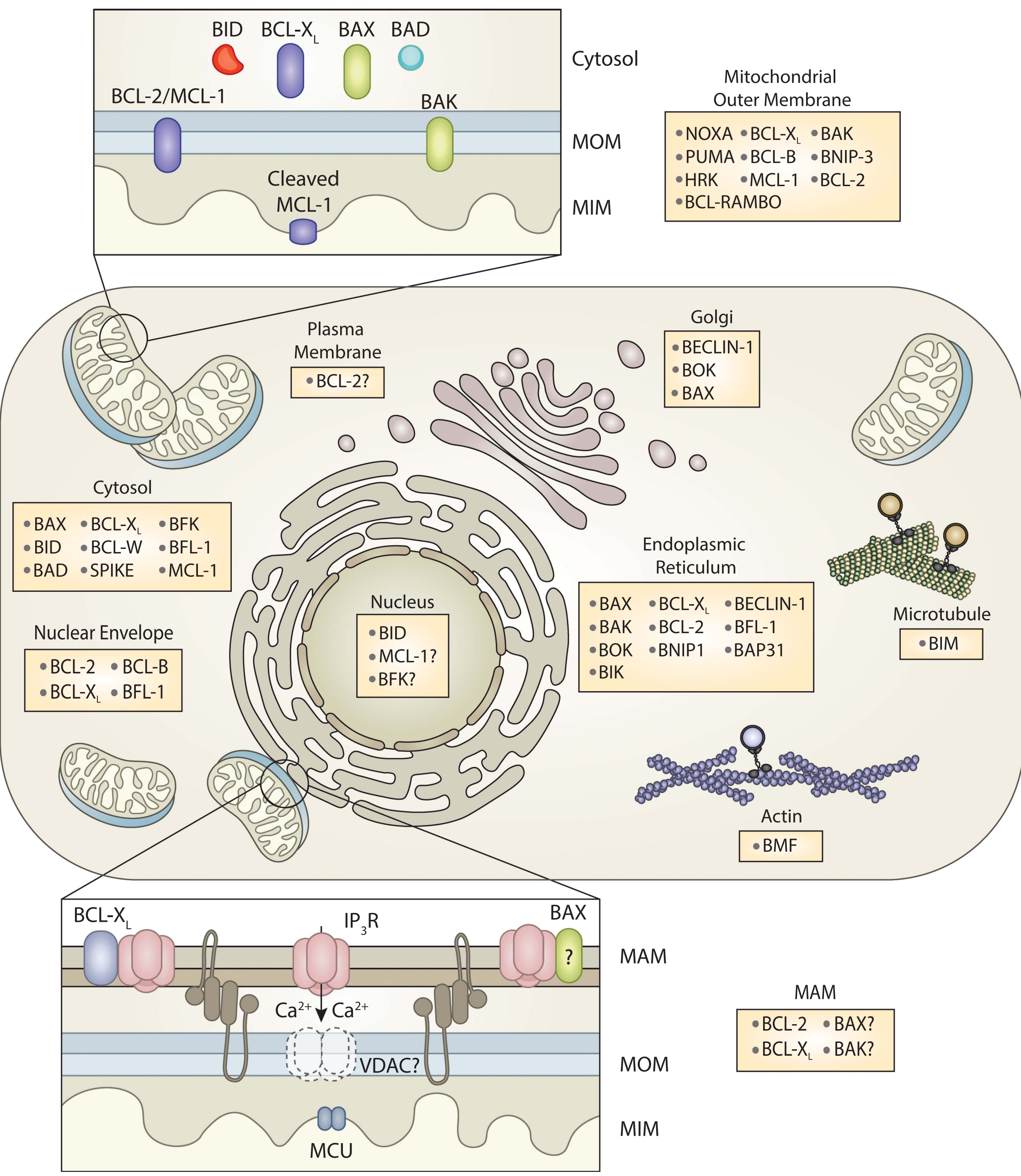


Figure 5 BCL-2 family protein subcellular localization in non-apoptotic cells BCL-2 family proteins reported at each location are listed in the corresponding box. Uncertainty or data for which there are conflicting reports are indicated by a '?'. Localization that changes during apoptosis is summarized in Tables 3A and 3B. Interactions at the mitochondria are shown in the enlargement top left. Interactions at the mitochondrial associated membrane (MAM), a subdomain of the endoplasmic reticulum in close contact with mitochondria are enlarged below left. Among other proteins, mitofusin proteins (shown as dimer linking the two membranes) bring a specialized subdomain of ER membranes in contact with the mitochondrial outer membrane forming the MAM

Table 3A Localization of BCL-2 Family proteins

	Localization									
Protein	ER	NE	TGN	Cytosol	MOM	MM	MIM	Cyto-skeleton	Plasma membrane	Nucleus
BCL-2	XX				XX					
BFL-1										
BCL-X_L										
					XX					
BCL-W										
MCL-1					XX					
					XX					
BCL-G					XX					
BAX				XX						
					XX					
BAK					XX					
BOK	XX									
BMF										
BIM										
BID				XX						
PUMA					XX					
NOXA					XX					
					XX					
HRK										
BAD				XX						
BIK	XX									
BECLIN-1	XX									
BNIP										
SPIKE				XX						
BAP31	XX									
BCL-G										
BFK				XX						
BCL-Rambo					XX					

Abbreviations

ER Endoplasmic Reticulum
NE Nuclear Envelope
MOM Mitochondrial Outer Membrane
MIM Mitochondrial Inner Membrane
MM Mitochondrial Matrix
TGN trans Golgi network

normal conditions

apoptotic conditions

XX predominant localization

Heat map of reported localizations for BCL-2 family proteins Proteins reported in multiple locations or that change location in growth and apoptotic conditions are shaded light and dark gray, respectively. XX indicates predominant localization for proteins reported at multiple locations

Table 3B Detailed localization information and references

	Senior Author	Species	Year	Localization: Resting	Localization: Apoptosis	Modification(s) affect localization
BCL-2 (B-cell lymphoma 2)						
8402648	JC Reed	Human	1993	ER, NE, & MOM	no change	
8043515	A Strasser	Human	1994			
8144576	DW Andrews	Human	1994			
BCL-X_L (B-cell lymphoma-extra large)						
9108035	RJ Youle	Murine	1997	Cytosol and MOM	MOM, ER & NE	
12783855	X Wang	Human	2003			
22544066	JT Opferman	Human and Murine	2012			
BCL-W / BCL2-L-2 (BCL-2-like protein 2)						
12952938	DCS Huang	Murine	2003	cytosol & loose association at MOM	C-terminal inserted MOM	
BFL-1(human) / A1(murine) (BCL-2-related protein A1)						
11929871	J Borst	Human	2002	ER, NE, & MOM	no change	
23499873	C Ferran	Human	2013			
16094403	C Gélinas	Human	2005			
MCL-1 (Induced myeloid leukemia cell differentiation protein)						
24525728	H Harada	Human	2014	MOM, Cytosol and ER	MOM	
7896880	RW Craig	Human	1995			
15798198	HFY Yen	Murine	2005			
22544066	JT Opferman	Human and Murine	2012		MOM, MM, MIM	N-terminal proteolytic cleavage
20627101	HFY Yen	Murine	2010	MIM		
15554878	V Duronio	Human and Murine	2005	Nucleus		protealytic cleavage
BCL-B / BCL2-L-10 (BCL-2-like protein 10)						
11278245	JC Reed	Human	2000	MOM	no change	
23563182	J Borst	Human	2013			
22233804	H Varmus	Human	2011		target to cytosol	Mono-Ubiquitination
BAX (BCL-2 Associated X)						
9382873	RJ Youle	Human	1997	Cytosol & Golgi	MOM, & ER	
9670005	SJ Korsemeyer	Murine	1998			
12847083	CB Thompson	Murine	2003			
22560721	M Deshmukh	Human	2012			
10228148	RJ Youle	Human	1999	translocation blocked		S184E
19376889	AB Waxman	Human	2009			Phosphorylation (S184)
BAK (Bcl-2 homologous antagonist/killer)						
12847083	CB Thompson	Murine	2003	MOM & ER	no change	
10085290	JA Hickman	Human	1999			
BOK (BCL-2 related ovarian killer)						
23429263	T Kaufmann	Murine	2013	ER & Golgi, MOM	no change	
27053113	JH Wojcikiewicz	Human	2016			
26949185	DR Green	Murine	2016			
BMF (BCL-2-modifying factor)						
14561217	MG Hinds	Murine	2004	Associated with (DLC)-2 (Actin Cytoskeleton)	MOM	Phosphorylation, release from actin cytoskeleton
12591950	K Lei & RJ Davis	Human	2003			
BIM-L and EL (BCL-2-interacting mediator of cell death), BIM-S= constitutively mitochondria						
17517961	G Hacker	Human	2015	Associated with (DLC)-1 (microtubules)	MOM	
12142566	Y Fukuuchi	Murine	2002			
12591950	RJ Davis	Human	2003			Phosphorylation, release from microtubules
BID (BH3 interacting-domain death agonist)						
10807576	N Tanaka	Murine	2000	Cytosol	MOM	Cleavage required for translocation
14500711	TH Kim	Human	2003			
PUMA\BBC3 (p53 upregulated modulator of apoptosis)						
11463392	KH Vousden	Human	2001	MOM	no change	
17968660	KH Vousden	Human	2008			
12574499	L Zhang	Human	2002			
26431330	A Vasquez	Human	2015	cytosol	MOM	
26212789	H You	Human	2015			Phosphorylation (S10) promotes translocation to MOM
NOXA						
10807576	N Tanaka	Murine	2000	MOM cytosol	MOM	Phosphorylation (S13) targets to cytosol
14500711	TH Kim	Human	2003			
21145489	A Kelekar	Human	2010			
HRK (Harakiri)						
15031724	C Kitanaka	Human	2004	not transcribed	MOM	
17434443	J Villalain	Human	2007			
BAD (BCL-2-associated death promoter)						
19641507	SJ Korsemeyer	Murine	2003	Cytosol (bound to 14-3-3) MOM	MOM	
16603546	UR Rapp	Human	2006			Dephosphorylation (S136), release Bad from 14-3-3, translocates to MOM
10230394	SJ Korsemeyer	Murine	1999			
10195903	JC Reed	Human	1999			
BIK (BCL-2 Interacting Killer)						
18299962	S Ge	Human	2008	ER	no change	
11884414	G Shore	Human	2002			
BECLIN-1 (Coiled-coil myosin-like BCL2-interacting protein)						
11306555	T Yoshimori	Human	2000	ER, TGN & MOM	no change	
16179260	B. Levine	Human	2005			
BNIP (BCL2/adenovirus E1B 19 kd-interacting protein)						
15272311	M Tagaya	Human	2004	BNIP1, 2: ER BNIP3: MOM	no change	
7954800	G Chinnadurai	Human	1994			
23896122	J. Tang	Human	2013			poly-ubiquitination relocates BNip1 to MOM
SPIKE						
20361936	M. Mitrovic	Human	2010	cytosol	no change	
BAP31 (B-cell receptor-associated protein 31)						
9334338	GC Shore	Human	1997	ER	no change	
12668660	GC Shore	Human	2003			
BCL-G (BCL-2-like protein 14)						
11054413	JC Reed	Human	2001	BCL-G_L : diffuse BCL-G_S : MOM	no change	
BFK (BCL-2 family kin)						
12700646	A Strasser	Human	2003	Cytosol	no change	
17412810	M Poutanen	Murine	2007	Nucleus Diffuse	no change	
BCL-RAMBO						
22921587	JH Park	Human	2012	MOM	no change	

Legend

(dark triangle)	Human	NE	Nuclear Envelope
(light triangle)	Murine	MOM	Mitochondrial Outer Membrane
(both)	Human and Murine	MIM	Mitochondrial Inner Membrane
(DLC)-1	Dynein Light Chain-1	TGN	trans Golgi network
(DLC)-2	Dynein Light Chain-2	underlined	denotes predominant localization
ER	Endoplasmic Reticulum		

binding partners at each location (Figure 5, Tables 3A and 3B). The localization of BCL-2 family proteins results from complex binding equilibria with membranes and other proteins within the cell. Localization dictates the available binding partners and therefore BCL-2 family response to different cell death stimuli. The subcellular localization of BCL-2 family proteins may also be important for monitoring various cellular processes and/or may be requisite to alternate functions performed by the proteins in non-apoptotic cells.

For example, detachment from the matrix and some drug treatments result in perturbations of the cytoskeleton that in many cell types causes a specialized form of apoptosis called anoikis. In response to anoikis BIM and BMF have been reported to translocate from the cytoskeleton, their location in non-apoptotic cells, to the MOM (Tables 3A and 3B,Figure 5). Moreover, loss of either protein results in protection from anoikis and resistance to cytoskeleton altering drugs like paclitaxel.[95,96] It seems likely therefore, that localization of BIM and BMF at the cytoskeleton permits monitoring cellular fitness and initiation of an apoptotic signal in response to cytoskeletal perturbation.

The most widely recognized non-mitochondrial localization for BCL-2 family proteins is the endoplasmic reticulum (ER) where they regulate calcium homeostasis, ER stress, autophagy and apoptosis.[97] The mechanism by which BCL-2 proteins regulate ER stress and calcium homeostasis and how this relates to apoptosis is not clear.[98] BCL-X_L, BCL-2, MCL-1, BAX and BAK have all been reported to regulate calcium release from the ER by interacting with inositol tri-phosphate receptors (IP3R), a ligand-gated calcium channel.[98] There is accumulating evidence that this interaction is mediated by the BH4 domain of the multi-domain anti-apoptotic proteins.[99] Moreover, BCL-2 and BCL-X_L can localize at the mitochondria-associated ER membranes, with Type III IP3Rs favouring transmission of calcium to the mitochondria, reducing calcium stores (Figure 5, panel B inset).[100,101] Although many details remain unclear, it has been hypothesized that anti-apoptotic proteins promote the slow leak of calcium from the ER, decreasing the potential for strong signaling to mitochondria and thereby inhibiting apoptosis.[101] BAX/BAK and BH3-only proteins may oppose calcium leakage by binding BCL-2/BCL-X_L or IP3R, increasing calcium stores and promoting apoptosis.[102,103] However, there are many areas of controversy and additional models for the role of BCL-2 family proteins in calcium signaling and ER stress.[98] Nevertheless, the preponderance of evidence suggests that BCL-2 family proteins function differently at the ER and mitochondria.

Using Small Molecules with High Affinities to BCL-2 Family Proteins to Control the Dance of Death

Developing small molecule modulators of BCL-2 family proteins has been a high priority for major pharmaceutical companies for more than a decade. Many cancer cells are 'addicted' to the expression of anti-apoptotic BCL-2 family proteins because in their absence the cells die faster than they grow. Such cells are described as 'primed for death' requiring only a 'push' in the right direction to trigger MOMP and

subsequent apoptosis.[104] Inactivating the anti-apoptotic BCL--2 family proteins with small molecule BH3 mimetic drugs is one potential ‘push’ as displacing the active but sequestered pro-apoptotic proteins results in MOMP.[9,105,106] Small molecule BH3 mimetics, like ABT-263 (Navitoclax) and ABT-199 (Venetoclax), mimic the binding of BH3 peptides to the hydrophobic BH3 domain-binding groove of anti-apoptotic proteins and thus displace BH3-only proteins and active BAX/BAK from anti-apoptotic proteins.[107,108] By binding to the BH3 domain-binding grooves of anti-apoptotic proteins, ABT-263 inhibits BCL-2, BCL-XL and BCL-W, whereas ABT-199 only inhibits BCL-2. ABT-199 is approved for use in chronic lymphocytic leukemia and both drugs are being used in dozens of clinical trials as single agents and in combination with other therapies.[109]

Some cancers depend primarily on MCL-1 for survival[110–112] and others acquire resistance to drugs that target BCL-2/BCL-XL/BCL-W by upregulating MCL-1.[113] The small molecule MCL-1 inhibitor, S63845 shows promise as a therapeutic.[114] S63845 was efficacious in killing multiple cancer-derived cell lines *in vitro* and had potent anti-tumor activity in pre-clinical mouse models of hematological malignancies *in vivo* while sparing normal tissues. Another cancer treatment strategy would be small molecule activation of BAX and/or BAK. However, it has yet to be determined whether such small molecules have any therapeutic index in cancer.[53]

ABT-263 and ABT-199 were optimized to displace BAD BH3 peptides from C-terminally truncated anti-apoptotic proteins in solution. However, the significant differences in BCL-2 family interactions between full-length proteins at membranes compared with truncated proteins in solution described above extend to interactions with drugs. For example, even though ABT-263 and the functionally similar BH3 mimetic ABT-737 displace BID and BAD, unlike what was seen *in vitro* using peptides and truncated proteins, in live cells the compounds do not displace BIM from BCL-2 or BCL-X_L.[115,116] Recently, this result was independently confirmed for BIM with the BCL-2 inhibitor ABT-199 and BCL-X_L inhibitor WEHI-539 and extended to include resistance in live cells of PUMA interactions with full-length BCL-2 and BCL-X_L, but not C-terminally truncated cytoplasmic anti-apoptotic proteins.[117] These studies are consistent with the tenets of the embedded together model in which membrane binding alters the functional interactions of BCL-2 family proteins via conformation changes that alter binding affinities. It has not been determined whether membrane binding, additional interactions outside of the BH3 domains or both contribute to the apparent enhanced affinity between these BCL-2 family proteins at membranes. However, these studies highlight the need to examine BCL-2 family interactions, and their pharmacological manipulation, in live cells.

Modeling the dance of death

Pharmacological manipulation of the BCL-2 family activities will be limited until there is a more quantitative understanding of how BCL-2 family proteins regulate apoptosis in cells. Quantitative modeling in combination with experimental data can be used to explain, predict and understand the behavior of complex biological processes while validating and discriminating competing models. Quantitative modeling was successfully used to explain the substantial cell-to-cell variability in cell death kinetics upon induction of extrinsic apoptosis.[5] A similar approach using Bayesian statistics discriminated between the competing models of BCL-2 family interactions by incorporating previously published binding affinities between BCL-2 family proteins.[118] This study concluded that only the ‘embedded together’ model reproduces MOMP dynamics observed in single-cells. These quantitative models provided important insights into the complex regulation of the BCL-2 family proteins that likely would have been missed otherwise. The large number of BCL-2 family proteins and differences in their binding affinities and mechanisms of action make predicting the apoptotic response of cells very difficult. We expect additional fine tuning of quantitative models will require inclusion of all (or most) of the determinants discussed in this review such as the range of concentrations of BCL-2 family proteins and their various affinities and binding partners in cells. As dysregulation of BCL-2 family proteins has wide-ranging implications in human disease, as difficult as it will be, quantitatively modeling the ‘dance of death’ by the Bcl-2 family proteins will be required to understand how apoptosis can be modulated to most effectively treat human disease.

Conflict of Interest

The authors declare no conflict of interest.

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1. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* 2010; **11**: 621–632.
2. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* 2015; **7**: 4.
3. Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol* 2000; **2**: 156–162.
4. Rehm M, Dussmann H, Janicke RU, Tavare JM, Kogel D, Prehn JH. Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3. *J Biol Chem* 2002; **277**: 24506–24514.
5. Albeck JG, Burke JM, Spencer SL, Lauffenburger DA, Sorger PK. Modeling a snap-action, variable-delay switch controlling extrinsic cell death. *PLoS Biol* 2008; **6**: 2831–2852.
6. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. *Oncogene* 2008; **27**: S2–19.
7. Shamas-Din A, Brahmbhatt H, Leber B, Andrews DW. BH3-only proteins: orchestrators of apoptosis. *Biochim Biophys Acta* 2011; **1813**: 508–520.
8. Billen LP, Shamas-Din A, Andrews DW. Bid: a Bax-like BH3 protein*Oncogene* 2009; **27**: S93–104.
9. Shamas-Din A, Kale J, Leber B, Andrews DW. Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harb Perspect Biol* 2013; **5**: a008714.
10. Leber B, Lin J, Andrews DW. Embedded together: the life and death consequences of interaction of the Bcl-2 family with membranes. *Apoptosis* 2007; **12**: 897–911.
11. Moldoveanu T, Grace CR, Llambi F, Nourse A, Fitzgerald P, Gehring K *et al.* BID-induced structural changes in BAK promote apoptosis. *Nat Struct Mol Biol* 2013; **20**: 589–597.
12. Czabotar PE, Westphal D, Dewson G, Ma S, Hockings C, Fairlie WD *et al.* Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell* 2013; **152**: 519–531.
13. Liu Q, Moldoveanu T, Sprules T, Matta-Camacho E, Mansur-Azzam N, Gehring K. Apoptotic regulation by MCL-1 through heterodimerization. *J Biol Chem* 2010; **285**: 19615–19624.
14. Ku B, Liang C, Jung JU, Oh BH. Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX. *Cell Res* 2011; **21**: 627–641.

15. Petros AM, Nettesheim DG, Wang Y, Olejniczak ET, Meadows RP, Mack J *et al.* Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. *Protein Sci* 2000; **9**: 2528–2534.
16. Chi X, Kale J, Leber B, Andrews DW. Regulating cell death at, on, and in membranes. *Biochim Biophys Acta* 2014; **1843**: 2100–2113.
17. Shamas-Din A, Bindner S, Zhu W, Zaltsman Y, Campbell C, Gross A *et al.* tBid undergoes multiple conformational changes at the membrane required for Bax activation. *J Biol Chem* 2013; **288**: 22111–22127.
18. Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE, Oltvai ZN. Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Semin Cancer Biol* 1993; **4**: 327–332.
19. Billen LP, Kokoski CL, Lovell JF, Leber B, Andrews DW. Bcl-XL inhibits membrane permeabilization by competing with Bax. *PLoS Biol* 2008; **6**: e147.
20. Lovell JF, Billen LP, Bindner S, Shamas-Din A, Fradin C, Leber B *et al.* Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. *Cell* 2008; **135**: 1074–1084.
21. Wang ZX. An exact mathematical expression for describing competitive binding of two different ligands to a protein molecule. *FEBS Lett* 1995; **360**: 111–114.
22. Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M *et al.* Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 1997; **275**: 983–986.
23. Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB. Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol Cell Biol* 1997; **17**: 7040–7046.
24. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; **17**: 393–403.
25. Petros AM, Medek A, Nettesheim DG, Kim DH, Yoon HS, Swift K *et al.* Solution structure of the antiapoptotic protein bcl-2. *Proc Natl Acad Sci USA* 2001; **98**: 3012–3017.
26. Czabotar PE, Lee EF, Thompson GV, Wardak AZ, Fairlie WD, Colman PM. Mutation to Bax beyond the BH3 domain disrupts interactions with pro-survival proteins and promotes apoptosis. *J Biol Chem* 2011; **286**: 7123–7131.
27. Polster BM, Basanez G, Young M, Suzuki M, Fiskum G. Inhibition of Bax-induced cytochrome c release from neural cell and brain mitochondria by dibucaine and propranolol. *J Neuroscience* 2003; **23**: 2735–2743.
28. Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R *et al.* Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. *J Cell Biol* 1998; **143**: 217–224.
29. Dutta S, Gulla S, Chen TS, Fire E, Grant RA, Keating AE. Determinants of BH3 binding specificity for Mcl-1 versus Bcl-xL. *J Mol Biol* 2010; **398**: 747–762.
30. Cheng C, Liu Y, Balasis ME, Garner TP, Li J, Simmons NL *et al.* Marinopyrrole derivatives with sulfide spacers as selective disruptors of Mcl-1 binding to pro-apoptotic protein Bim. *Marine Drugs* 2014; **12**: 4311–4325.
31. Smith AJ, Dai H, Correia C, Takahashi R, Lee SH, Schmitz I *et al.* Noxa/Bcl-2 protein interactions contribute to bortezomib resistance in human lymphoid cells. *J Biol Chem* 2011; **286**: 17682–17692.
32. Hockings C, Anwari K, Ninnis RL, Brouwer J, O'Hely M, Evangelista M *et al.* Bid chimeras indicate that most BH3-only proteins can directly activate Bak and Bax, and show no preference for Bak versus Bax. *Cell Death Dis* 2015; **6**: e1735.
33. Merino D, Giam M, Hughes PD, Siggs OM, Heger K, O'Reilly LA *et al.* The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *J Cell Biol* 2009; **186**: 355–362.
34. Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE *et al.* Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* 2007; **315**: 856–859.
35. Du H, Wolf J, Schafer B, Moldoveanu T, Chipuk JE, Kuwana T. BH3 domains other than Bim and Bid can directly activate Bax/Bak. *J Biol Chem* 2011; **286**: 491–501.
36. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR *et al.* BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005; **17**: 525–535.
37. Sarosiek KA, Chi X, Bachman JA, Sims JJ, Montero J, Patel L *et al.* BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. *Mol Cell* 2013; **51**: 751–765.
38. Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD *et al.* Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science* 2004; **305**: 1466–1470.
39. Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG *et al.* BAX activation is initiated at a novel interaction site. *Nature* 2008; **455**: 1076–1081.
40. Walensky LD, Pitter K, Morash J, Oh KJ, Barbuto S, Fisher J *et al.* A stapled BID BH3 helix directly binds and activates BAX. *Mol Cell* 2006; **24**: 199–210.
41. Wilfling F, Weber A, Potthoff S, Vogtle FN, Meisinger C, Paschen SA *et al.* BH3-only proteins are tail-anchored in the outer mitochondrial membrane and can initiate the activation of Bax. *Cell Death Differ* 2012; **19**: 1328–1336.
42. Lutter M, Fang M, Luo X, Nishijima M, Xie X, Wang X. Cardiolipin provides specificity for targeting of tBid to mitochondria. *Nat Cell Biol* 2000; **2**: 754–761.
43. Kim TH, Zhao Y, Ding WX, Shin JN, He X, Seo YW *et al.* Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome C release. *Mol Biol Cell* 2004; **15**: 3061–3072.
44. Oh KJ, Barbuto S, Pitter K, Morash J, Walensky LD, Korsmeyer SJ. A membrane-targeted BID BCL-2 homology 3 peptide is sufficient for high potency activation of BAX in vitro. *J Biol Chem* 2006; **281**: 36999–37008.
45. Birkinshaw RW, Czabotar PE. The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. *Semin Cell Dev Biol* 2017 (in press).
46. Llambi F, Moldoveanu T, Tait SW, Bouchier-Hayes L, Temirov J, McCormick LL *et al.* A unified model of mammalian BCL-2 protein family interactions at the mitochondria. *Mol Cell* 2011; **44**: 517–531.
47. Cosentino K, Garcia-Saez AJ. Bax and Bak pores: are we closing the circle? *Trends Cell Biol* 2017; **27**: 266–275.
48. Garner TP, Reyna DE, Priyadarshi A, Chen HC, Li S, Wu Y *et al.* An autoinhibited dimeric form of BAX regulates the BAX activation pathway. *Mol Cell* 2016; **64**: 431.
49. Westphal D, Dewson G, Menard M, Frederick P, Iyer S, Bartolo R *et al.* Apoptotic pore formation is associated with in-plane insertion of Bak or Bax central helices into the mitochondrial outer membrane. *Proc Natl Acad Sci USA* 2014; **111**: E4076–E4085.
50. Shamas-Din A, Bindner S, Chi X, Leber B, Andrews DW, Fradin C. Distinct lipid effects on tBid and Bim activation of membrane permeabilization by pro-apoptotic Bax. *Biochem J* 2015; **467**: 495–505.
51. Weber A, Paschen SA, Heger K, Wilfling F, Frankenberg T, Bauerschmitt H *et al.* BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptotic Bcl-2 proteins. *J Cell Biol* 2007; **177**: 625–636.
52. Iyer S, Anwari K, Alsop AE, Yuen WS, Huang DC, Carroll J *et al.* Identification of an activation site in Bak and mitochondrial Bax triggered by antibodies. *Nat Commun* 2016; **7**: 11734.
53. Brahmbhatt H, Uehling D, Al-Awar R, Leber B, Andrews D. Small molecules reveal an alternative mechanism of Bax activation. *Biochem J* 2016; **473**: 1073–1083.
54. Pritz JR, Wachter F, Lee S, Luccarelli J, Wales TE, Cohen DT *et al.* Allosteric sensitization of proapoptotic BAX. *Nat Chem Biol* 2017; **13**: 961–967.
55. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tu S, Beere HM *et al.* The multidomain proapoptotic molecules Bax and Bak are directly activated by heat. *Proc Natl Acad Sci USA* 2005; **102**: 17975–17980.
56. Cartron PF, Oliver L, Mayat E, Meflah K, Vallette FM. Impact of pH on Bax alpha conformation, oligomerisation and mitochondrial integration. *FEBS Lett* 2004; **578**: 41–46.
57. Hsu YT, Youle RJ. Nonionic detergents induce dimerization among members of the Bcl-2 family. *J Biol Chem* 1997; **272**: 13829–13834.
58. Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R *et al.* Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002; **111**: 331–342.
59. Yethon JA, Epand RF, Leber B, Epand RM, Andrews DW. Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis. *J Biol Chem* 2003; **278**: 48935–48941.
60. Chipuk JE, McStay GP, Bharti A, Kuwana T, Clarke CJ, Siskind LJ *et al.* Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. *Cell* 2012; **148**: 988–1000.
61. O'Neill KL, Huang K, Zhang J, Chen Y, Luo X. Inactivation of prosurvival Bcl-2 proteins activates Bax/Bak through the outer mitochondrial membrane. *Genes Dev* 2016; **30**: 973–988.
62. Suzuki M, Youle RJ, Tjandra N. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 2000; **103**: 645–654.
63. Setoguchi K, Otera H, Mihara K. Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins. *EMBO J* 2006; **25**: 5635–5647.
64. Nechushtan A, Smith CL, Hsu YT, Youle RJ. Conformation of the Bax C-terminus regulates subcellular location and cell death. *EMBO J* 1999; **18**: 2330–2341.
65. Robin AY, Krishna Kumar K, Westphal D, Wardak AZ, Thompson GV, Dewson G *et al.* Crystal structure of Bax bound to the BH3 peptide of Bim identifies important contacts for interaction. *Cell Death Dis* 2015; **6**: e1809.
66. Dai H, Smith A, Meng XW, Schneider PA, Pang YP, Kaufmann SH. Transient binding of an activator BH3 domain to the Bak BH3-binding groove initiates Bak oligomerization. *J Cell Biol* 2011; **194**: 39–48.
67. Bleicken S, Jeschke G, Stegmueller C, Salvador-Gallego R, Garcia-Saez AJ, Bordignon E. Structural model of active Bax at the membrane. *Mol Cell* 2014; **56**: 496–505.
68. Brouwer JM, Westphal D, Dewson G, Robin AY, Uren RT, Bartolo R *et al.* Bak core and latch domains separate during activation, and freed core domains form symmetric homodimers. *Mol Cell* 2014; **55**: 938–946.
69. Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP *et al.* Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell* 2009; **36**: 487–499.
70. Annis MG, Soucie EL, Dlugosz PJ, Cruz-Aguado JA, Penn LZ, Leber B *et al.* Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis. *EMBO J* 2005; **24**: 2096–2103.
71. Zhang Z, Subramaniam S, Kale J, Liao C, Huang B, Brahmbhatt H *et al.* BH3-in-groove dimerization initiates and helix 9 dimerization expands Bax pore assembly in membranes. *EMBO J* 2016; **35**: 208–236.
72. Mandal T, Shin S, Aluvila S, Chen HC, Grieve C, Choe JY *et al.* Assembly of Bak homodimers into higher order homooligomers in the mitochondrial apoptotic pore. *Sci Rep* 2016; **6**: 30763.
73. Subburaj Y, Cosentino K, Axmann M, Pedrueza-Villalmanzo E, Hermann E, Bleicken S *et al.* Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species. *Nat Commun* 2015; **6**: 8042.

74. Dewson G, Kratina T, Czabotar P, Day CL, Adams JM, Kluck RM. Bak activation for apoptosis involves oligomerization of dimers via their alpha6 helices. *Mol Cell* 2009; **36**: 696–703.
75. Dewson G, Ma S, Frederick P, Hockings C, Tan I, Kratina T *et al.* Bax dimerizes via a symmetric BH3:groove interface during apoptosis. *Cell Death Differ* 2012; **19**: 661–670.
76. Satsoura D, Kucerka N, Shivakumar S, Pencer J, Griffiths C, Leber B *et al.* Interaction of the full-length Bax protein with biomimetic mitochondrial liposomes: a small-angle neutron scattering and fluorescence study. *Biochim Biophys Acta* 2012; **1818**: 384–401.
77. Gahl RF, Dwivedi P, Tjandra N. Bcl-2 proteins bid and bax form a network to permeabilize the mitochondria at the onset of apoptosis. *Cell Death Dis* 2016; **7**: e2424.
78. Bleicken S, Classen M, Padmavathi PV, Ishikawa T, Zeth K, Steinhoff HJ *et al.* Molecular details of Bax activation, oligomerization, and membrane insertion. *J Biol Chem* 2010; **285**: 6636–6647.
79. Niu X, Brahmbhatt H, Mergenthaler P, Zhang Z, Sang J, Daude M *et al.* A small-molecule inhibitor of Bax and Bak oligomerization prevents genotoxic cell death and promotes neuroprotection. *Cell Chem Biol* 2017; **24**: 493–506 e5.
80. Ding J, Mooers BH, Zhang Z, Kale J, Falcone D, McNichol J *et al.* After embedding in membranes antiapoptotic Bcl-XL protein binds both Bcl-2 homology region 3 and helix 1 of proapoptotic Bax protein to inhibit apoptotic mitochondrial permeabilization. *J Biol Chem* 2014; **289**: 11873–11896.
81. Wieckowski MR, Vyssokikh M, Dymkowska D, Antonsson B, Brdiczka D, Wojtczak L. Oligomeric C-terminal truncated Bax preferentially releases cytochrome c but not adenylate kinase from mitochondria, outer membrane vesicles and proteoliposomes. *FEBS Lett* 2001; **505**: 453–459.
82. Bleicken S, Landeta O, Landajuela A, Basanez G, Garcia-Saez AJ. Proapoptotic Bax and Bak proteins form stable protein-permeable pores of tunable size. *J Biol Chem* 2013; **288**: 33241–33252.
83. Gillies LA, Du H, Peters B, Knudson CM, Newmeyer DD, Kuwana T. Visual and functional demonstration of growing Bax-induced pores in mitochondrial outer membranes. *Mol Biol Cell* 2015; **26**: 339–349.
84. Salvador-Gallego R, Mund M, Cosentino K, Schneider J, Unsay J, Schraermeyer U *et al.* Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores. *EMBO J* 2016; **35**: 389–401.
85. Kuwana T, Olson NH, Kiosses WB, Peters B, Newmeyer DD. Pro-apoptotic Bax molecules densely populate the edges of membrane pores. *Sci Rep* 2016; **6**: 27299.
86. Kutuk O, Letai A. Regulation of Bcl-2 family proteins by posttranslational modifications. *Curr Mol Med* 2008; **8**: 102–118.
87. Vantieghem A, Xu Y, Assefa Z, Piette J, Vandenheede JR, Merlevede W *et al.* Phosphorylation of Bcl-2 in G2/M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal and delays the onset of apoptosis. *J Biol Chem* 2002; **277**: 37718–37731.
88. Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol* 1999; **19**: 8469–8478.
89. Geng F, Tang L, Li Y, Yang L, Choi KS, Kazim AL *et al.* Allyl isothiocyanate arrests cancer cells in mitosis, and mitotic arrest in turn leads to apoptosis via Bcl-2 protein phosphorylation. *J Biol Chem* 2011; **286**: 32259–32267.
90. Deng X, Gao F, Flagg T, May WS Jr. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *Proc Natl Acad Sci USA* 2004; **101**: 153–158.
91. Deng X, Ruvolo P, Carr B, May WS Jr. Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc Natl Acad Sci USA* 2000; **97**: 1578–1583.
92. Ruvolo PP, Deng X, Carr BK, May WS. A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem* 1998; **273**: 25436–25442.
93. Linseman DA, Butts BD, Precht TA, Phelps RA, Le SS, Laessig TA *et al.* Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. *J Neurosci* 2004; **24**: 9993–10002.
94. Gardai SJ, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N *et al.* Phosphorylation of Bax Ser(184) by Akt regulates its activity and apoptosis in neutrophils. *J Biol Chem* 2004; **279**: 21085–21095.
95. Li R, Moudgil T, Ross HJ, Hu HM. Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim. *Cell Death Differ* 2005; **12**: 292–303.
96. Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE *et al.* Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* 2001; **293**: 1829–1832.
97. Leber B, Geng F, Kale J, Andrews DW. Drugs targeting Bcl-2 family members as an emerging strategy in cancer. *Expert Rev Mol Med* 2010; **12**: e28.
98. Lewis A, Hayashi T, Su TP, Betenbaugh MJ. Bcl-2 family in inter-organelle modulation of calcium signaling; roles in bioenergetics and cell survival. *J Bioenerg Biomembr* 2014; **46**: 1–15.
99. Monaco G, Vervliet T, Akl H, Bultynck G. The selective BH4-domain biology of Bcl-2-family members: IP3Rs and beyond. *Cell Mol Life Sci* 2013; **70**: 1171–1183.
100. Williams A, Hayashi T, Wolozny D, Yin B, Su TC, Betenbaugh MJ *et al.* The non-apoptotic action of Bcl-xL: regulating Ca(2+) signaling and bioenergetics at the ER-mitochondrion interface. *J Bioenerg Biomembr* 2016; **48**: 211–225.
101. Li C, Wang X, Vais H, Thompson CB, Foskett JK, White C. Apoptosis regulation by Bcl-x(L) modulation of mammalian inositol 1,4,5-trisphosphate receptor channel isoform gating. *Proc Natl Acad Sci USA* 2007; **104**: 12565–12570.
102. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T *et al.* BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* 2003; **300**: 135–139.
103. Chami M, Prandini A, Campanella M, Pinton P, Szabadkai G, Reed JC *et al.* Bcl-2 and Bax exert opposing effects on Ca2+ signaling, which do not depend on their putative pore-forming region. *J Biol Chem* 2004; **279**: 54581–54589.
104. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA *et al.* Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 2006; **9**: 351–365.
105. Del Gaizo Moore V, Letai A. BH3 profiling–measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. *Cancer Lett* 2013; **332**: 202–205.
106. Leber B, Lin J, Andrews DW. Still embedded together binding to membranes regulates Bcl-2 protein interactions. *Oncogene* 2010; **29**: 5221–5230.
107. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S *et al.* ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* 2008; **68**: 3421–3428.
108. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J *et al.* ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* 2013; **19**: 202–208.
109. Delbridge AR, Grabow S, Strasser A, Vaux DL. Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. *Nat Rev Cancer* 2016; **16**: 99–109.
110. Grabow S, Delbridge AR, Valente LJ, Strasser A. MCL-1 but not BCL-XL is critical for the development and sustained expansion of thymic lymphoma in p53-deficient mice. *Blood* 2014; **124**: 3939–3946.
111. Zhang H, Guttikonda S, Roberts L, Uziel T, Semizarov D, Elmore SW *et al.* Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. *Oncogene* 2011; **30**: 1963–1968.
112. Xiang Z, Luo H, Payton JE, Cain J, Ley TJ, Opferman JT *et al.* Mcl1 haploinsufficiency protects mice from Myc-induced acute myeloid leukemia. *J Clin Invest* 2010; **120**: 2109–2118.
113. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* 2010; **115**: 3304–3313.
114. Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G *et al.* The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature* 2016; **538**: 477–482.
115. Liu Q, Leber B, Andrews DW. Interactions of pro-apoptotic BH3 proteins with anti-apoptotic Bcl-2 family proteins measured in live MCF-7 cells using FLIM FRET. *Cell Cycle* 2012; **11**: 3536–3542.
116. Aranovich A, Liu Q, Collins T, Geng F, Dixit S, Leber B *et al.* Differences in the mechanisms of proapoptotic BH3 proteins binding to Bcl-XL and Bcl-2 quantified in live MCF-7 cells. *Mol Cell* 2012; **45**: 754–763.
117. Pecot J, Maillet L, Le Pen J, Vuillier C, Trecesson SC, Fetiveau A *et al.* Tight Sequestration of BH3 Proteins by BCL-xL at Subcellular Membranes Contributes to Apoptotic Resistance. *Cell Rep* 2016; **17**: 3347–3358.
118. Lopez CF, Muhlich JL, Bachman JA, Sorger PK. Programming biological models in Python using PySB. *Mol Syst Biol* 2013; **9**: 646.

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